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AMRL-TR-76-80

MUTAGEN AND ONCOGEN STUDY OF METHYLHYDRAZINE FINAL REPORT

LITTON BIONETICS, INC.
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KENSINGTON, MARYLAND 20795

DECEMBER 1976

AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
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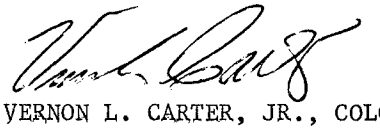

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AIR FORCE - 17 JANUARY 77 - 100

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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: AMRL-TR-76-80	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE *(and Subtitle)*: MUTAGEN AND ONCOGEN STUDY OF METHYLHYDRAZINE	5. TYPE OF REPORT & PERIOD COVERED: FINAL
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): David Brusick, Ph.D., Dale W. Matheson, Ph.D.	8. CONTRACT OR GRANT NUMBER(s): F33615-76-C-0515
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Litton Bionetics, Incorporated, 5516 Nicholson Lane, Kensington, MD 20795	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 61102F, 2312, 2312V1, 2312V115
11. CONTROLLING OFFICE NAME AND ADDRESS: Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Sys Cmd, Wright-Patterson Air Force Base, Ohio 45433	12. REPORT DATE: DECEMBER 1976 13. NUMBER OF PAGES: 38
14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*	15. SECURITY CLASS. *(of this report)*: Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Methylhydrazine
Mutagenesis
DNA Repair
Microsome Activation

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

A three tier test was organized into a matrix of assays employing microbial cells, mammalian cells in culture, and in vivo experiments in rats and mice. Methylhydrazine (MH) was mutagenic for Salmonella typhimurium TA-1535 in suspension tests. Results from all other assays conducted as part of this evaluation were considered to be negative.

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PREFACE

This research was initiated by the Toxicology Branch, Toxic Hazards Division, Aerospace Medical Research Laboratory. Experiments were performed under Contract F33615-76-C-0515 by Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20795.

The experiments were conducted by David Brusick, Ph.D., and Dale W. Matheson, Ph.D., of Litton Bionetics, Inc., Kensington, Maryland 20795. Kenneth C. Back, Ph.D., was contract monitor for the Aerospace Medical Research Laboratory.

1. INTRODUCTION

The detection and subsequent confirmation of mutagenic substances capable of producing germ cell mutations requires a multifaceted testing program. The components of such a program should be able to detect both point mutations and chromosomal aberrations since these two classes of genetic alterations represent the types of transmissible mutations that are of concern to man. The tests included in a mutagenicity evaluation program for chemicals should not only be sensitive and reproducible, but also relevant to normal exposure and pharmacological conditions encountered in the environment. These latter two conditions are often difficult to achieve since good human model systems are lacking. It may be argued that if a single toxicologic end point, e.g., mutation, can be demonstrated in several different test species, then application of the response to a wide range of species, including man, can be made. Therefore, a mutagenicity evaluation program should contain a series of assays covering many phylogenetic levels.

LBI feels that the program conducted in this study offered as many of the essential test criteria as possible for an accurate evaluation of methylhydrazine (MH) for genetic activity. Selected tests from Tiers I, II, and III were organized into a matrix of assays employing microbial cells, mammalian cells in culture, and *in vivo* experiments in rats and mice.

Tests utilizing these organisms measured point mutations (forward and reverse), chromosomal aberrations, and mitotic recombinational events induced by acute and subchronic exposure to the test substance.

Figure 1 illustrates the composition of the test program prepared for the genetic evaluation of the test substance. A brief summary of each of the assays is listed as follows:

A. *In Vitro Microbial Assays*

In these assays, the test substance was evaluated for mutagenic and recombinogenic activity in strains of *Salmonella* and *Saccharomyces*, respectively. Metabolic activation of the compound was obtained by combining hepatic microsomes with the test system. Nonactivation and activation semiquantitative plate tests were conducted.

B. *In Vitro Mutation Assay in Mammalian Cells*

In this assay, the mutations were measured in cultured mouse cells (L5178Y). Both direct and *in vitro* activation assays were performed. The specific event detected by these cells was forward mutation at the thymidine kinase ($TK^{+/-} \rightarrow TK^{-/-}$) locus, which is an autosomal recessive trait. The combined *in vitro* tests from A and B gave a very sensitive measurement of the test substance's ability to induce point mutations and mitotic recombination.

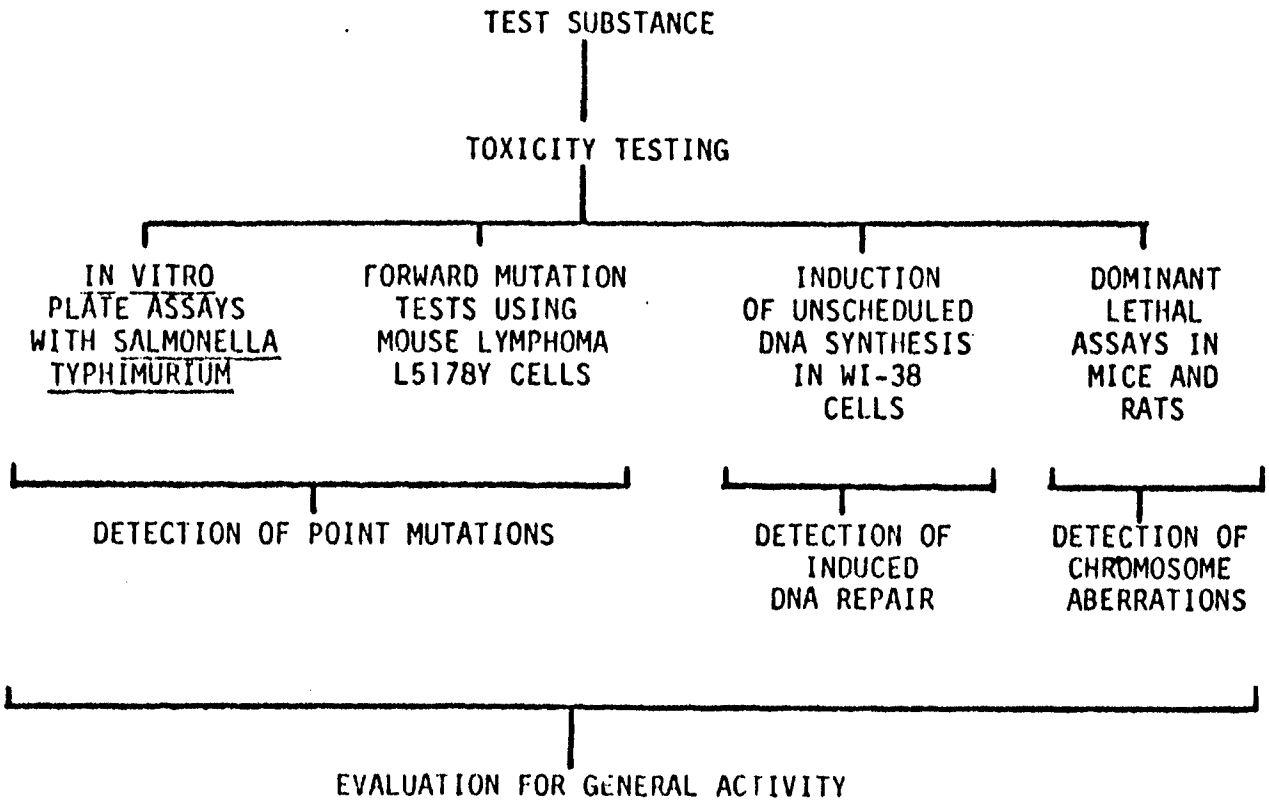

FIGURE 1

COMPOSITION OF THE GENETIC EVALUATION PROGRAM

C. Unscheduled DNA Synthesis

A second component of the in vitro mammalian cell assay system utilized the human diploid WI-38 strain of cells. This cell strain, obtained from human embryonic lung, was used to measure test chemical-induced DNA repair in cells not undergoing scheduled (S phase) DNA synthesis.

Normal DNA synthesis occurs in the S phase of the cell cycle with little or no synthesis occurring in any of the other phases (G_0, G_1, G_2, or M). The detection of significant DNA synthesis during these stages (UDS) is indicative of the stimulation of repair enzyme systems. Exposure of WI-38 cells to various forms of radiation or to chemicals known to be mutagenic or carcinogenic has resulted in the stimulation of UDS (1).

The detection of UDS in WI-38 cells involved exposure of the cells to the test chemical followed by the addition of tritiated thymidine (^{3}H-TdR) to the culture. If DNA damage has been induced, the ^{3}H-TdR will be incorporated during the repair of the DNA. This incorporation can be detected by scintillation counting.

D. Dominant Lethal Assay

This assay was designed to determine the ability of a compound to induce genetic damage to the germ cells of treated male mice and rats leading to fetal wastage. Chromosome aberrations, including breaks, rearrangements, and deletions, are believed to produce the dominant lethality. Male mice and rats were exposed to several dose levels of the test compound for five days and then sequentially mated to two virgin untreated females each week over the period of spermatogenesis. At mid-pregnancy the females were killed and scored with respect to the number of living and dead implants as well as to the level of fertility. These results were then compared to data from control animals.

E. Background

Hydrazines and MH react with pyrimidine bases, especially at high pH, breaking the pyrimidine ring and causing the removal of the base from DNA (2). Based on this type of reaction with DNA, the potential for hydrazine and/or derivatives to exhibit mutagenic, teratogenic, and carcinogenic activity might be expected.

Some information on the mutagenic and carcinogenic properties of hydrazine and some of its derivatives has been published.

Hydrazine in relatively high doses causes leukemia, reticulum cell sarcoma, and lung adenomas in mice (3). Hydrazine is mutagenic in T4 phage (4), S. typhimurium (5) and Drosophila melanogaster (D. melanogaster) (6), but was not active in a dominant lethal assay (7). Symetrical dimethylhydrazine induced mitotic gene conversion (8).

Because the test agent is structurally related to chemicals that are established mutagens and carcinogens, and because of the excellent correlation between mutagenicity and carcinogenicity, mutagenesis studies might provide insight into potential toxicologic problems associated with the test agent.

2. MATERIALS

A. In Vitro Microbial Assays

The test chemical was examined in a series of microbial assays employing histidine-requiring mutants of S. typhimurium. The assays were conducted so that the compound was tested directly and in the presence of a mouse liver microsome activation system.

The compound was evaluated at a minimum of four dose levels under both test conditions with the highest dose level showing some evidence of toxicity. In addition to these tests, spot tests (5) were conducted with the Salmonella mutants plus additional strains of bacteria; S. typhimurium strain G-46 and E. coli strain WP_2uvrA^- (11).

1. Preparation of Tissue Homogenates and 9,000 x g Cell Fractions

Male mice (sufficient to provide the necessary quantities of tissues) were killed by cranial blow, decapitated, and bled. Organs were immediately dissected from the animal using aseptic techniques and placed in ice-cold 0.25 M sucrose buffered with Tris buffer at a pH of 7.4. Upon collection of the desired quantity of organs, they were washed twice with fresh buffered sucrose and completely homogenized with a motor-drive homogenizing unit at 4C. The whole organ homogenate obtained from this step was centrifuged for 20 minutes at 9,000 x g in a refrigerated centrifuge. The supernatant from the centrifuged sample was retained and frozen at -80C. Samples from these preparations were used for the activation studies.

2. Reaction Mixture

The following reaction mixture was employed in the activation tests:

Component	Final Concentration/ml
TPN (sodium salt)	6 μM
Isocitric acid	35 μM
Tris buffer, pH 7.4	28 μM
$MgCl_2$	2 μM
Homogenate fraction equivalent to 25 mg of wet tissue	

3. Solvent and Control Compounds

Preparation and dilution of test compounds were done in dimethylsulfoxide (DMSO). Positive control compounds were included as reference points and to ensure that the assay was functioning with known mutagens. Direct acting mutagens were employed in nonactivation assays and mutagens requiring microsomal activation were used in activation assays. The compounds and the concentrations employed are provided in the data tables.

4. Bacteria Cultures

Overnight cultures of S. typhimurium G-46, TA-1535, TA-1537, TA-1538, TA-98, and TA-100 were employed along with E. coli strain WP_2uvrA^- and S. cerevisiae strain D4. All cultures were monitored regularly for stability of markers and contamination.

B. In Vitro Mutation Assay in Mammalian Cells

The test chemical was tested for mutagenic activity in a forward mutation assay employing cultured mouse cells (L5178Y). The cell line is heterozygous for the thymidine kinase ($TK^{+/-}$) gene and the assay detects homozygous $TK^{-/-}$ mutant clones. The compounds were tested directly and in the presence of a mouse liver microsome activation system.

1. Preparation of Tissue Homogenates and 9,000 x g Cell Fractions: The activation system employed in this assay was the same as described for the Microbial Assays.

2. Reaction Mixture: The same reaction mixture as described for the Microbial Assays was used for these studies.

3. Solvent and Control Compounds: Preparation of stock chemicals was done in DMSO. All dilutions of test chemicals were made in F_{10p} culture medium. Positive control mutagens active directly and requiring microsome activation were employed with all tests.

4. Cells and Media: $TK^{+/-}$ BUdR-sensitive L5178Y mouse lymphoma cells were used in this assay. Growth medium (GM) for this line consists of Fischer's mouse leukemia medium supplemented with 10% horse serum and sodium pyruvate (F_{10p}). Cloning medium consists of Fischer's medium plus 20% horse serum and agar (0.37%). Selective medium for $TK^{-/-}$ cells was prepared by adding BUdR to the cloning medium.

C. Unscheduled DNA Synthesis (UDS) Assay

Nondividing WI-38 cells were exposed to three concentrations of the test compound and ^{3}H-thymidine. Treatment was direct and under conditions of microsome activation. The amount of ^{3}H-thymidine incorporated into the DNA was measured by scintillation counting.

1. Preparation of Mouse Liver Microsomes

A 9,000 x g supernatant of mouse liver was prepared as described in the Microbial Assays. This supernatant was then centrifuged at 105,000 x g for 60 minutes and the pelleted microsomes resuspended in 0.25 M sucrose. This microsome preparation was added to the reaction mixture in place of the 9,000 x g cell fraction.

2. Reaction Mixture

The reaction mixture was the same as used in the Microbial Assays except purified microsomes replaced the 9,000 x g supernatant.

3. Solvent and Control Compounds

Any stock solutions of chemicals were prepared and diluted in DMSO. Positive control chemicals that act directly and require microsome activation were employed.

4. Cells and Media

Human diploid embryonic lung cells (WI-38) were obtained from Flow Laboratories and used in these assays. The GM employed was Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS). Step-down medium (SM) was amino acid depleted to reduce cell division, and hydroxyurea medium (HUM) was the medium used to inhibit S phase growth. All media were based on EMEM.

D. Dominant Lethal Assay (DLA)

The test chemical was tested in mouse and rat DLA. All animals were dosed by intraperitoneal (IP) injections over five consecutive days, rested for two days, and mated.

1. Animals

a. Mice: Seven- to eight-week-old male random bred mice (ICR, Flow) were used for treatment. Female mice of the same strain, age, and weight were used for the matings.

b. Rats: Ten- to twelve-week-old Sprague-Dawley male rats from a random bred closed colony (Flow) were used for treatment. Females of the same strain, age, and weight were used for the matings.

2. Animal Husbandry

Each species was housed in separate rooms of our animal facility.

Male mice were housed five to a cage while being dosed with the compound, and then housed separately with two females for mating.

All animals were offered a 4% fat diet and water ad libitum. Water was acidified according to approved laboratory animal health standards.

Animals were identified by ear punch. Sanitary cages and bedding were used and changed two times per week at which times water containers were cleaned, sanitized, and filled. Cages were repositioned on racks once a week, and the racks repositioned within rooms monthly. Personnel handling animals or working with animal facilities wear head and face masks as well as suitable garments. Individuals with respiratory or other overt infections are excluded from the animal facility.

3. Positive and Negative Control Chemicals

Triethylenemelamine (TEM) was administered IP at a level of 0.3 mg/kg in 0.85% saline as a positive control. Negative control animals received an IP injection of the corn oil or water solvents.

E. Test Chemicals

The test sample was obtained from the United States Air Force. MH was a clear liquid (less than 100 ml) in an amber bottle.

3. METHODS

A. In Vitro Microbial Assays

Overnight cultures of S. typhimurium TA-1535, TA-1537, TA-1538, TA-98, TA-100, E. coli WP_2uvrA^-, and S. cerevisiae D4 were grown in complete broth. Approximately 10^8 cells from a culture were added to test tubes containing 2.0 ml of molten agar supplemented with biotin and a trace amount of histidine.

Four dose levels of the test chemical were added to the appropriate tubes and the contents poured over selective medium. In activation tests 0.5 ml aliquots of the reaction mixture containing the microsomes were added to the tubes containing cells and chemical just prior to pouring onto the selective medium. After the overlays solidified, the plates were placed in a 37C incubator for 48 to 72 hours. The plates were then scored for the number of colonies growing in the agar overlay. Positive and solvent controls using both direct-acting mutagens and promutagens that required metabolic activation were run with each assay. Supplementary spot tests were also conducted according to the methods described by Ames et al. (12).

The data are presented in Table 1. Concentrations of the test and positive control chemicals are given in the data tables.

B. In Vitro Mutation Assay in Mammalian Cells

1. Toxicity

The solubility, toxicity, and doses for the test chemical were determined prior to screening. The effect of the chemical on the survival of the indicator cells was determined by exposing the cells to a wide range of chemical concentrations in complete GM. Toxicity was measured as loss in growth potential of the cells induced by a five-hour exposure to the chemical. Four doses were selected from the range of concentrations by using the highest dose that showed no loss in growth potential as the penultimate dose and by bracketing this with one higher dose and two lower doses. Toxicity produced by chemical treatment was monitored during the experiment.

2. Test

a. Nonactivation assay

The procedure used was a modification of that reported by Clive and Spector (13). Prior to each treatment, cells were cleansed of spontaneous TK$^{-/-}$ by growing them in a medium containing thymidine, hypoxanthine, methotrexate, and glycine (THMG). This medium permits the survival of only those cells that produce the enzyme thymidine kinase, and can therefore utilize the exogenous thymidine from the medium. The test compound was added to the cleansed cells in GM at the predetermined doses for five hours. The mutagenized cells were washed, fed, and allowed to express in GM for three days. At the end of this expression period, TK$^{-/-}$ mutants were detected by cloning the cells in the selection medium for ten days. Surviving cell populations were determined by plating diluted aliquots in nonselective GM.

b. Activation Assay

The activation assay differs from the nonactivation assay in the following manner only. Two and five tenths ml of the reaction mixture was added to 10 ml of GM. The desired number of cleansed cells was added to this mixture, and the flask was incubated on a rotary shaker for five hours. The incubation period was terminated by washing the cells twice with GM. The washed mutagenized cells were then allowed to express for three days and were cloned as indicated for the nonactivated cells.

c. Data Analysis

A mutation frequency for each test dose was determined by dividing the number of mutants/ml by the number of surviving cells/ml (adjusted to 10^{-4}) as indicated by plating efficiency. These data are presented in Table 2. Concentrations of the test and positive control chemicals are given in the data tables.

C. Unscheduled DNA Synthesis

1. Cell Preparation

Normal human diploid WI-38 cells were seeded at 5×10^5 cells in a 100 mm tissue culture dish and grown to confluency in GM. Once reaching confluency, the cells were switched to SM for five days. The contact inhibition imposed by confluency and the use of SM held the cells in a nonproliferating state.

2. Treatment

On the day of treatment, cells held in G_1 phase were placed in HUM. After 30 minutes this medium was replaced by 5 ml of HUM containing the control or test chemical and 1.0 µCi of ^{3}HTdR. Each treatment was at three concentrations. Exposure was terminated by washing the cells twice in cold BSS containing an excess of cold thymidine.

3. DNA Extraction and Measurement of ^{3}HTdR Incorporation

Treated plates were frozen at -20C until processed. After thawing, the cells on the 100 mm plate were covered with 2.5% SDS in 1 x SSC and scraped from the dish with a rubber policeman. The cells were washed and precipitated from the SDS by three changes of 95% ethanol and centrifuged at 10,000 x g. Additional lipid components were removed by extraction in ethanol ether at 70C. This pellet was washed in 70% ethanol, further incubated at 70C in 0.3N NaOH, and the DNA extracted in 50 µl 1N PCA at 70C. The DNA was separated into two 25 µl aliquots. One of these was dissolved in 10 ml of hydromix scintillation cocktail (Yorktown Co.) and counted in a Beckman liquid scintillation spectrometer. The second aliquot was added to 275 µl of 1N PCA and read at 260 nm in a Gilford spectrophotometer. The values were corrected for light scatter and converted to µg of DNA. Following liquid scintillation counting, the data were combined with the DNA extraction values and expressed as disintegration per minute per µg DNA (DPM/µg DNA).

4. Activation Assays

The activation tests were conducted according to the methods described above except that 0.62 ml of a purified microsome preparation (100,000 x g pellet) was added to the test mixture.

5. Dosage Determinations

Doses were determined from preliminary toxicity tests in which cells were seeded in 16 mm wells (Linbro plate). A wide range of concentrations was tested in the wells, and toxicity was monitored visually by altered cell morphology and adhesion. The three doses used in the experiments were selected.

The results of these tests are given in Table 3. The concentrations of test and control compounds are given in the data tables.

D. Dominant Lethal Assay

The dominant lethal assay is designed to assess the ability of the test compound or its metabolic products to reach the testes of treated male animals and induce genetic activity in the developing gametes during spermatogenesis.

MH was administered to male mice weighing 30 ± 2.5 gm. In addition, MH was also dosed to male rats weighing 325 ± 25 gm.

1. Stock Solutions

The compound was prepared daily from stock solutions. MH was dissolved in distilled water.

2. Compound Administration

Dosages were determined from LD50 data supplied by the contract monitor with a high dose of 1/10 the LD50, an intermediate dose at 1/3 the high level, and a low dose of 1/10 the high level. Compound was injected IP into each animal daily for five days. All dosages and routes of administration were determined in consultation with Dr. Kenneth Back of the United States Air Force.

Calculated dosages are as follows:

Methylhydrazine

Mice

LD50	26.0	mg/kg
High 1/10 LD50	2.6	mg/kg
Int. 1/30 LD50	0.86	mg/kg
Low 1/100 LD50	0.26	mg/kg

Rats

LD50	21.5	mg/kg
High 1/10 LD50	2.15	mg/kg
Int. 1/30 LD50	0.72	mg/kg
Low 1/100 LD50	0.215	mg/kg

3. Animal Husbandry

a. Mice: Ten male mice were housed five animals to a cage during the five days of dosing. After two days of rest, each male was caged with two virgin females from Monday through Friday. This sequence was repeated weekly with two new females each week for eight weeks. Fourteen days from the midweek in

which they were caged with the males, females were killed, dissected, and the number of dead, living, and total embryos in the uterus recorded on standard forms. These data were statistically analyzed for indications of dominant lethality, and compared with control data for significance.

b. Rats: The protocol for the experiment using rats differed from that of the mice only in the sequence of mating lasting seven weeks and in that corpora lutea were counted and recorded. These scores were used to determine evidence of compound-induced preimplantation losses.

4. Data

The results of the Dominant Lethal Assay are given in Tables 4 to 17.

4. RESULTS

The results of the genetic studies are presented in the following series of tables:

TABLE 1

RESULTS FROM MICROBIAL ASSAYS EVALUATING THE GENETIC ACTIVITY OF MH

Concentration (µl/plate)	Revertants Per Plate with Indicator Strain						
	TA-1535	TA-1537	TA-1538	TA-98	TA-100	D4	WP_2uvrA^-
Nonactivation							
Solvent Control	23(-)	18(-)	21(-)	17(-)	117(-)	31(-)	(-)
Positive Control	$>10^3$(+)	$>10^3$(+)	$>10^3$(+)	440(+)	$>10^3$(+)	172(+)	(+)
MH							
0.0001	16	13	15	25	68	-	-
0.001	27	13	17	21	143	31	-
0.01	20	23	15	22	143	33	-
0.1	16	28	12	12	128	39	-
1.0	0	0	0	0	92	61	(-)
5.0	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	(-)
Activation							
Solvent Control	32(-)	21(-)	30(-)	72(-)	89(-)	33(-)	(-)
Positive Control	$>10^3$(+)	240(+)	$>10^3$(+)	$>10^3$(+)	$>10^3$(+)	-	(+)
MH							
0.01	31	38	29	48	64	36	-
0.1	33	25	28	51	68	32	-
1.0	41	52	19	56	44	30	-
5.0	31(-)	44	19(-)	65(-)	93(-)	34(-)	(-)

( ) = Results of qualitative spot test (+) = positive response
(-) = negative response

TABLE 1A

Results From Suspension Tests of MH Using
S. typhimurium Strain TA-1535[a]

Test	Compound Concentration/ml	Population Counts	Mutant Counts	Mutation Freq. ($x10^{-8}$)
Activation				
Solvent Control (a)	-	2698*	227	8.4
(b)	-	1369**	86	6.3
Positive Control	DMN 100 µmoles	1264	4798	379.6
MH	1 µl	1043**	332	31.8
MH	5 µl	1958*	9855	503.3

*

**Identifies treated group with appropriate solvent control.

[a]The suspension assay was conducted using the same mouse liver activation system described for the plate assays shown in Table 1. The protocol was changed such that rather than add all test components to semisolid overlay agar, they were suspended in saline and incubated 60 minutes at 37°C on a rotary shaker. After incubation samples were removed and assayed for the numbers of surviving cells and numbers of revertants. Mutation frequencies were calculated for each test. Dimethylnitrosamine (DMN) was used as the positive control compound.

KEY

MOUSE LYMPHOMA ASSAY TABLE

COLUMN		
A, B, C, D	Day	= Expression day cell counts (x 10^6)
E	ΔGS	= Represents cell population growth during expression. The value is obtained by subtracting the Day 1 counts from the terminal day counts.
F	%GS	= Percent suspension growth is obtained by expressing the ΔGS values for treated cells as a percent of the ΔGS for the negative controls $\frac{\text{E treated}}{\text{E control}} \times 100$
G	MC	= Mutant counts. The total number of colonies counted in the BUdR plates.
H	VC	= Viable counts. The total number of colonies counted in the VC plates.
I	%CE	= Cloning efficiency $\frac{\text{VC counts in treated cultures}}{\text{VC counts in control cultures}} \times 100$
J	GF	= Growth factor $\frac{\text{Percent suspension growth (column F)} \times \text{Percent clonal growth (column I)}}{100}$
K	MF(x 10^{-4})	= Mutation frequency $\frac{\text{MC counts (column G)}}{\text{VC counts (column H)}} \times 10^{-4}$

TABLE 2

RESULTS OF THE MOUSE LYMPHOMA MUTAGENICITY ASSAY FOR MH

Test	A Day 1	B 2	C 3	D 4	E ΔGS	F % GS	G MC	H VC	I % CE	J GF	K MF(10^{-4})
Nonactivation											
Solvent Control	0.1	-	1.1	2.9	2.8	100	137	112	100	100	1.2
Positive Control	1.6	-	2.3	-	0.7	7	288	7	4	0.3	41.1
MH											
0.0005 µl/ml	1.5	-	3.2	5.8	4.3	153	58	48	43	65	1.2
0.001 µl/ml	1.3	-	1.6	6.7	5.4	192	120	63	56	107	1.9
0.05 µl/ml	1.8	-	2.7	6.1	4.3	154	87	60	54	82	1.5
0.1 µl/ml	1.8	-	2.2	3.6	1.8	64	102	92	82	58	1.1
Activation											
Solvent Control	4.7	-	10.6	-	5.9	100	22	81	100	100	0.3
Positive Control	1.5	-	0.2	-	0.2	3	184	8	3	0.9	2.3
MH											
0.001 µl/ml	2.9	-	15.8	-	13.9	235	7	65	80	188	0.1
0.005 µl/ml	4.2	-	13.0	-	9.8	166	11	42	52	86	0.3
0.01 µl/ml	4.3	-	7.4	-	3.1	53	5	16	20	10	0.3
0.05 µl/ml	3.0	-	3.7	-	0.7	12	6	12	15	2	0.5

TABLE 3

MEASUREMENT OF UDS IN WI-38 CELLS TREATED WITH MH

Test	Concentration (µl/ml)	DNA(µg)	DPM	Activity Index[a]	Percent of Control[b]
Nonactivation					
Solvent Control	-	9.02	76	8.4	-
Positive Control	MNNG (10µg/ml)	1.76	86	48.9	582
MH	0.1	9.64	102	10.6	126
	0.5	16.88	83	4.9	58
	1.0	9.25	102	10.4	124
Activation					
Solvent Control	-	13.64	78	5.7	-
Positive Control	2AAF (30µg/ml)	2.45	60	24.5	430
MH	0.1	22.56	145	6.4	112
	0.5	14.85	79	5.3	93
	1.0	Sample Lost			

[a]Activity Index = DPM/µg DNA (DPM = Disintegrations/minute)

[b]Percent of Control = $\frac{\text{Activity Index Treated}}{\text{Activity Index Control}} \times 100$

TABLE 4

COMPOUND MH STUDY SUBACUTE/MICE

FERTILITY INDEX

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.26 MG/KG	DOSE LEVEL 0.86 MG/KG	DOSE LEVEL 2.60 MG/KG	POSITIVE CONTROL
		1	6/ 20=0.30	3/ 20=0.15	5/ 20=0.25	4/ 19=0.21	2/ 20=0.10
$	$	2	13/ 20=0.65	10/ 20=0.50	10/ 20=0.50	5/ 20=0.25*	5/ 20=0.25*
		3	11/ 20=0.55	8/ 20=0.40	10/ 20=0.50	9/ 20=0.45	5/ 20=0.25
		4	14/ 20=0.70	9/ 20=0.45	11/ 19=0.58	12/ 17=0.71	14/ 20=0.70
		**					
		6	8/ 15=0.53	12/ 20=0.60	12/ 19=0.63	9/ 18=0.50	13/ 16=0.81
		7	10/ 20=0.50	5/ 19=0.26	13/ 20=0.65	6/ 18=0.33	11/ 20=0.55
		8	6/ 20=0.30	9/ 20=0.45	10/ 20=0.50	8/ 18=0.44	10/ 20=0.50

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE $,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
$ SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)
**Week 5 data not analyzed because of low fertility among all groups of animals

TABLE 5

COMPOUND MH STUDY SUBACUTE/MICE

AVERAGE NUMBER OF IMPLANTATIONS PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.26 MG/KG	DOSE LEVEL 0.86 MG/KG	DOSE LEVEL 2.60 MG/KG	POSITIVE CONTROL
		1	67/ 6=11.2	35/ 3=11.7	58/ 5=11.6	46/ 4=11.5	16/ 2= 8.0
		2	155/ 13=11.9	122/ 10=12.2	113/ 10=11.3	51/ 5=10.2	50/ 5=10.0@D
		3	133/ 11=12.1	116/ 8=14.5*@I	115/ 10=11.5	117/ 9=13.0	46/ 5= 9.2
		4	156/ 14=11.1	99/ 9=11.0	131/ 11=11.9	149/ 12=12.4	100/ 14=11.4
		5	97/ 8=12.1	148/ 12=12.3	153/ 12=12.8	115/ 9=12.8	183/ 13=14.1
		6	110/ 10=11.0	48/ 5= 9.6	156/ 13=12.0	63/ 6=10.5	117/ 11=10.6
		7	77/ 6=12.8	107/ 9=11.9	131/ 10=13.1	96/ 8=12.0	132/ 10=13.2

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
$ AND @ = ONE-TAILED TEST

ONE $,&,@,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,$ SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 6

COMPOUND MII STUDY SUBACUTE /MICE

AVERAGE RESORPTIONS (DEAD IMPLANTS) PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.26 MG/KG	DOSE LEVEL 0.86 MG/KG	DOSE LEVEL 2.60 MG/KG	POSITIVE CONTROL
		1	0/ 0=0.0	0/ 3=0.0	0/ 5=0.0	0/ 4=0.0	7/ 2=3.50
		2	7/ 13=0.54	6/ 10=0.60	7/ 10=0.70	0/ 5=0.0 **@@D	39/ 5=7.80**@@I
		3	6/ 11=0.55	0/ 8=0.0 *@D	4/ 10=0.40	5/ 9=0.56	20/ 5=4.00**@@I
		4	10/ 14=0.71	5/ 9=0.55	11/ 11=1.00	5/ 12=0.42	8/ 14=0.57
		5	4/ 8=0.50	5/ 12=0.42	5/ 12=0.42	5/ 9=0.56	7/ 13=0.54
		6	6/ 10=0.60	1/ 5=0.20	8/ 13=0.62	2/ 6=0.33	4/ 11=0.36
		7	4/ 6=0.67	6/ 9=0.67	4/ 10=0.40	6/ 8=0.75	1/ 10=0.10*@D

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
$ AND @ = ONE-TAILED TEST

ONE $,&,@,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,$ SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 7

COMPOUND MH STUDY SUBACUTE/MICE

PROPORTION OF FEMALES WITH ONE OR MORE DEAD IMPLANTATIONS

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.26 MG/KG	DOSE LEVEL 0.86 MG/KG	DOSE LEVEL 2.60 MG/KG	POSITIVE CONTROL
		1	0/ 6=0.0	0/ 3=0.0	0/ 5=0.0	0/ 4=0.0	2/ 2=1.00**
$	$	2	6/ 13=0.46	5/ 10=0.50	3/ 10=0.30	0/ 5=0.0	5/ 5=1.00*
		3	4/ 11=0.36	0/ 8=0.0	3/ 10=0.30	4/ 9=0.44	5/ 5=1.00*
		4	6/ 14=0.43	3/ 9=0.33	6/ 11=0.55	3/ 12=0.25	6/ 14=0.43
		5	2/ 8=0.25	5/ 12=0.42	4/ 12=0.33	5/ 9=0.56	5/ 13=0.38
		6	6/ 10=0.60	1/ 5=0.20	6/ 13=0.46	1/ 6=0.17	3/ 11=0.27
		7	4/ 6=0.67	4/ 9=0.44	3/ 10=0.30	4/ 8=0.50	1/ 10=0.10*

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE $,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
$ SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 8

COMPOUND MH STUDY SUBACUTE /MICE

PORPORTION OF FEMALES WITH TWO OR MORE DEAD IMPLANTATIONS

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.26 MG/KG	DOSE LEVEL 0.86 MG/KG	DOSE LEVEL 2.60 MG/KG	POSITIVE CONTROL
		1	0/ 6=0.0	0/ 3=0.0	0/ 5=0.0	0/ 4=0.0	2/ 2=1.00**
		2	1/ 13=0.08	1/ 10=0.10	2/ 10=0.20	0/ 5=0.0	5/ 5=1.00**
		3	1/ 11=0.09	0/ 8=0.0	1/ 10=0.10	1/ 9=0.11	4/ 5=0.80**
		4	3/ 14=0.21	2/ 9=0.22	4/ 11=0.36	1/ 12=0.08	2/ 14=0.14
		5	2/ 8=0.25	0/ 14=0.0	1/ 12=0.08	0/ 9=0.0	2/ 13=0.15
		6	0/ 10=0.0	0/ 5=0.0	1/ 13=0.08	1/ 6=0.17	1/ 11=0.09
		7	0/ 6=0.0	1/ 9=0.11	1/ 10=0.10	2/ 8=0.25	0/ 10=0.0

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE $,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
$ SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 9

COMPOUND NH STUDY SUBACUTE/MICE

DEAD IMPLANTS / TOTAL IMPLANTS

WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.26 MG/KG	DOSE LEVEL 0.86 MG/KG	DOSE LEVEL 2.60 MG/KG	POSITIVE CONTROL
1	0/ 67=0.0	0/ 35=0.0	0/ 58=0.0	0/ 46=0.0	7/ 16=0.44
2	7/155=0.05	6/122=0.05	7/113=0.06	0/ 51=0.0 *@D	39/ 50=0.78**@@1
3	6/133=0.05	0/116=0.0 *@D	4/115=0.03	5/117=0.04	20/ 46=0.43**@@1
4	10/156=0.06	5/ 89=0.05	11/131=0.08	5/149=0.03	8/160=0.05
5	4/ 97=0.04	5/148=0.03	5/153=0.03	5/115=0.04	7/183=0.04
6	6/110=0.05	1/ 40=0.02	8/156=0.05	2/ 63=0.03	4/117=0.03
7	4/ 77=0.05	6/107=0.06	4/131=0.03	6/ 96=0.06	1/132=0.01*@D

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT DIFFERENCES USING THE HISTORICAL CONTROL GROUP

* = TWO-TAILED TEST
@ = ONE-TAILED TEST

ONE *,@ = SIGNIFICANT AT P LESS THAN 0.05
TWO *,@ = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL

TABLE 10.
COMPOUND MH STUDY SUBACUTE/RATS

FERTILITY INDEX

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.215 MG/KG	DOSE LEVEL 0.720 MG/KG	DOSE LEVEL 2.150 MG/KG	POSITIVE CONTROL
		1	8/ 20=0.40	6/ 20=0.30	7/ 20=0.35	9/ 20=0.45	10/ 20=0.50
		2	11/ 20=0.55	13/ 20=0.65	13/ 20=0.65	14/ 20=0.70	15/ 20=0.75
$	$	3	8/ 20=0.40	9/ 20=0.45	13/ 20=0.65	14/ 20=0.70	9/ 20=0.45
		4	16/ 20=0.80	16/ 20=0.80	11/ 18=0.61	16/ 20=0.80	9/ 20=0.45*
		5	14/ 20=0.70	11/ 20=0.55	13/ 17=0.76	15/ 20=0.75	13/ 20=0.65
		6	15/ 20=0.75	12/ 20=0.60	15/ 18=0.83	15/ 20=0.75	12/ 20=0.60
		7	20/ 20=1.00	14/ 20=0.70**	16/ 18=0.89	18/ 20=0.90	14/ 20=0.70**

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE $,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
$ SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 11

COMPOUND MH STUDY SUBACUTE/RATS

AVERAGE NUMBER OF IMPLANTATIONS PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.215 MG/KG	DOSE LEVEL 0.720 MG/KG	DOSE LEVEL 2.150 MG/KG	POSITIVE CONTROL
		1	94/ 8=11.8	67/ 6=11.2	69/ 7= 9.9	93/ 9=10.3	97/ 10= 9.7
& $	$	2	104/ 11= 9.5	147/ 13=11.3	148/ 13=11.4	171/ 14=12.2*@1	70/ 15= 4.7**@@D
		3	100/ 8=12.5	116/ 9=12.8	176/ 13=13.5	174/ 14=12.4	47/ 9= 5.2**@@D
		4	188/ 16=11.8	207/ 16=12.9	144/ 11=13.1@1	212/ 16=13.3	30/ 9= 3.3**@@D
		5	148/ 14=10.6	148/ 11=13.5**@@	151/ 13=11.6	180/ 15=12.0	126/ 13= 9.8
		6	178/ 15=11.9	146/ 12=12.2	191/ 15=12.7	182/ 15=12.1	141/ 12=11.8
		7	255/ 20=12.8	167/ 14=11.9	168/ 16=11.8	236/ 18=13.1	144/ 14=10.3*@D

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
$ AND @ = ONE-TAILED TEST

ONE $,&,@,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,$ SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 12

COMPOUND MH STUDY SUBACUTE/RATS

AVERAGE CORPORA LUTEA PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.215 MG/KG	DOSE LEVEL 0.720 MG/KG	DOSE LEVEL 2.150 MG/KG	POSITIVE CONTROL
		1	111/ 8=13.9	77/ 6=12.8	86/ 7=12.3	111/ 9=12.3*ω0	121/ 10=12.1
$	$	2	134/ 11=12.2	167/ 13=12.8	169/ 13=13.0	201/ 14=14.4ω1	173/ 15=11.5
		3	123/ 8=15.4	123/ 9=13.7ω0	186/ 13=14.3	193/ 14=13.8ω0	95/ 9=10.6**ω0
$	$	4	198/ 16=12.4	217/ 16=13.6	150/ 11=13.6	228/ 16=14.3*ω1	103/ 9=11.4
		5	163/ 14=11.6	150/ 11=13.6**ω1	167/ 13=12.1	193/ 15=12.9	154/ 13=11.8
		6	186/ 15=12.4	149/ 12=12.4	202/ 15=13.5ω1	192/ 15=12.8	153/ 12=12.8
		7	313/ 20=15.6	217/ 14=15.5	264/ 16=16.9	267/ 18=14.8	200/ 14=14.3

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
$ AND ω = ONE-TAILED TEST

ONE $,&,ω,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,&,ω,* = SIGNIFICANT AT P LESS THAN 0.01

*,ω SIGNIFICANTLY DIFFERENT FROM CONTROL
&,$ SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 13

COMPOUND MII STUDY SUBACUTE/RATS

AVERAGE PREIMPLANTATION LOSSES PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.215 MG/KG	DOSE LEVEL 0.720 MG/KG	DOSE LEVEL 2.150 MG/KG	POSITIVE CONTROL
		1	17/ 8= 2.1	10/ 6= 1.7	11/ 7= 2.4	18/ 9= 2.0	24/ 10= 2.4
		2	30/ 11= 2.7	20/ 13= 1.5	21/ 13= 1.6	30/ 14= 2.1	103/ 15= 6.9**@@I
		3	23/ 8= 2.9	8/ 9= 0.9**@@D	10/ 13= 0.8**@@D	19/ 14= 1.4*@D	48/ 9= 5.3@I
		4	10/ 16= 0.6	10/ 16= 0.6	6/ 11= 0.5	16/ 16= 1.0	13/ 9= 8.1**@@I
		5	15/ 14= 1.1	2/ 11= 0.2@D	6/ 13= 0.5	13/ 15= 0.9	26/ 13= 2.0
		6	8/ 15= 0.5	3/ 12= 0.3	11/ 15= 0.7	10/ 15= 0.7	12/ 12= 1.0
$		7	58/ 20= 2.9	50/ 14= 3.6	66/ 16= 4.1	31/ 18= 1.7@D	56/ 14= 4.0

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

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$ AND @ = ONE-TAILED TEST

ONE $,&,@,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,$ SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 14

COMPOUND MH STUDY SUBACUTE/RATS

AVERAGE RESORPTIONS (DEAD IMPLANTS) PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.215 MG/KG	DOSE LEVEL 0.720 MG/KG	DOSE LEVEL 2.150 MG/KG	POSITIVE CONTROL
		1	6/ 8=0.75	4/ 6=0.67	3/ 7=0.43	2/ 9=0.22	15/ 10=1.50
		2	1/ 11=0.09	7/ 13=0.64	1/ 13=0.08	2/ 14=0.14	3/ 15=0.20
&$$	&$$	3	8/ 8=1.00	4/ 9=0.44	6/ 13=0.46	0/ 14=0.0 *@D	46/ 9=5.11**@@I
		4	10/ 16=0.63	8/ 16=0.50	7/ 11=0.64	16/ 16=1.00	28/ 9=3.11**@@I
$	$	5	7/ 14=0.50	4/ 11=0.36	12/ 13=0.92	19/ 15=1.27	85/ 13=6.54**@@I
		6	13/ 16=0.87	5/ 12=0.42	5/ 15=0.33	10/ 15=0.67	23/ 12=1.92@I
		7	0/ 20=0.0	10/ 14=0.71**@@I	8/ 16=0.50@I	6/ 18=0.33**@@I	5/ 14=0.36*@@I

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
$ AND @ = ONE-TAILED TEST

ONE $,&,@,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,$ SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 15

COMPOUND MH STUDY SUBACUTE/RATS

PROPORTION OF FEMALES WITH ONE OR MORE DEAD IMPLANTATIONS

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.215 MG/KG	DOSE LEVEL 0.720 MG/KG	DOSE LEVEL 2.150 MG/KG	POSITIVE CONTROL
		1	4/ 8=0.50	2/ 6=0.33	2/ 7=0.29	2/ 9=0.22	5/ 10=0.50
		2	1/ 11=0.09	4/ 13=0.31	1/ 13=0.08	2/ 14=0.14	3/ 15=0.20
$$	$$	3	4/ 8=0.50	4/ 9=0.44	4/ 13=0.31	0/ 14=0.0 **	9/ 9=1.00*
		4	6/ 16=0.38	6/ 16=0.38	5/ 11=0.45	10/ 16=0.63	9/ 9=1.00**
		5	6/ 14=0.43	4/ 11=0.36	5/ 13=0.38	8/ 15=0.53	13/ 13=1.00**
		6	6/ 15=0.40	5/ 12=0.42	4/ 15=0.27	4/ 15=0.27	9/ 12=0.75
		7	0/ 20=0.0	6/ 14=0.43**	3/ 16=0.19*	6/ 18=0.33**	5/ 14=0.36**

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE $,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,** = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
$ SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 16

COMPOUND MH STUDY SUBACUTE/RATS

PERCENTAGE OF FEMALES WITH TWO OR MORE DEAD IMPLANTATIONS

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.215 MG/KG	DOSE LEVEL 0.720 MG/KG	DOSE LEVEL 2.150 MG/KG	POSITIVE CONTROL
		1	2/ 8=0.25	1/ 6=0.17	1/ 7=0.14	0/ 9=0.0	3/ 10=0.30
		2	0/ 11=0.0	2/ 13=0.15	0/ 13=0.0	0/ 14=0.0	0/ 15=0.0
		3	2/ 8=0.25	0/ 9=0.0	2/ 13=0.15	0/ 14=0.0 *	9/ 9=1.00**
		4	3/ 16=0.19	2/ 16=0.13	1/ 11=0.09	3/ 16=0.19	6/ 9=0.67*
$$	$$	5	1/ 14=0.07	0/ 11=0.0	4/ 13=0.31	7/ 15=0.47*	13/ 13=1.00**
		6	4/ 15=0.27	0/ 12=0.0	1/ 15=0.07	3/ 15=0.20	7/ 12=0.58
		7	0/ 20=0.0	3/ 14=0.21*	3/ 16=0.19*	0/ 18=0.0	0/ 14=0.0

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE $,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
$ SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 17

COMPOUND MII STUDY SUBACUTE/RATS

DEAD IMPLANTS / TOTAL IMPLANTS

WEEK	NEGATIVE CONTROL	DOSE LEVEL 0.215 MG/KG	DOSE LEVEL 0.720 MG/KG	DOSE LEVEL 2.150 MG/KG	POSITIVE CONTROL
1	6/ 94=0.06	4/ 67=0.06	3/ 69=0.04	2/ 93=0.02	15/ 97=0.15
2	1/104=0.01	7/147=0.05	1/148=0.01	2/171=0.01	3/ 70=0.04*@1
3	8/100=0.08	4/115=0.03	6/176=0.03	0/174=0.0 *@0	46/ 47=0.98**@@1
4	10/186=0.05	8/201=0.04	7/144=0.05	16/212=0.08	28/ 30=0.93**@@1
5	7/148=0.05	4/148=0.03	12/151=0.08	19/180=0.11	85/128=0.66**@@1
6	13/178=0.07	5/146=0.03	5/191=0.03	10/182=0.05	23/141=0.16@1
7	0/255=0.0	10/167=0.06**@@1	8/188=0.04	6/236=0.03*@@1	5/144=0.03**@@1

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT DIFFERENCES USING THE HISTORICAL CONTROL GROUP

* = TWO-TAILED TEST
@ = ONE-TAILED TEST

ONE *,@ = SIGNIFICANT AT P LESS THAN 0.05
TWO *,@ = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL

5. INTERPRETATIONS AND CONCLUSIONS

MH was evaluated for its genetic activity and ability to stimulate DNA repair using a battery of *in vitro* and *in vivo* assays.

A. Microbial Assays (Table 1)

There were no clear indications of mutagenic activity by MH in any of the microbial assays reported in Table 1. The toxicity of MH for bacteria and yeast was high and concentrations of 10 µl/plate were consistently too toxic to use.

Because hydrazines have certain properties similar to nitrosamines, this compound was also examined for mutagenicity in a suspension assay. Dimethylnitrosamine was found to be inactive in the standard plate assay, but the chemical is highly mutagenic when tested in a suspension procedure. The results for MH under the same test conditions were also found to be positive (Table 1A). Mutagenic activity was observed at MH concentrations of 1 µl and 5 µl/ml after a 60-minute incubation with a mouse liver activation system.

B. Mouse Lymphoma Assays (Table 2)

The data from these tests were clearly negative.

C. UDS Assay in WI-38 Cells (Table 3)

The data from these tests were clearly negative. The sample cells at the high dose level (activation assay) were lost by breakage in the centrifuge. However, there was no indication of a trend or any activity in an equivalent dose in nonactivation tests.

D. Dominant Lethal Assays

Dominant lethality, which is indicated by a high percentage of dead implants to total implants, was not demonstrated by the data from either mice or rats administered MH. In comparison, the positive control compound TEM demonstrated a clear dominant lethal effect in mice during weeks 1 through 3 and in rats during weeks 1 through 5.

1. Mice (Tables 4-9)

Implant data for week 5 were not statistically analyzed due to the low number of pregnant females in the low and intermediate dosage groups. The reason for the low fertility in these animals is unknown, but does not appear to be compound related since the negative control group also only contained one pregnant animal. Implant data from the other weeks did not reveal any of the dose-related trends indicative of compound-induced genetic activity.

2. Rats (Tables 10-17)

Increased lethality compared to negative controls significant at $p < 0.01$ was indicated for week 7 dosages. The ratios of dead to total implants for these animals fall within the range of variation encountered for all levels of tests throughout the testing period; and, therefore, the statistical significance is associated with the week 7 negative control animals not having any dead implants. In addition, no dose-related trends were observed. In light of these observations and since the ratios are considerably less than those for weeks 1 through 5 of the positive controls, we did not consider the data as indicating biologically significant activity for MH in rats.

E. Conclusions

MH was mutagenic in activation, microbial reversion tests if the tests were conducted as suspension tests and not if conducted as standard plate tests. This differential activity is similar to the type of results obtained with dimethyl- and diethylnitrosamines. Except for the mutagenicity of MH in tests with S. typhimurium TA-1535, there were no indications of genetic activity for MH in any of the other tests conducted as part of this evaluation.

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